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14. ABSTRACT
Osteoblastic bone metastasis is a common complication of advanced prostate cancer, resulting in pain and pathologic fracture. Dickkopf homolog 1 (DKK1) is a secreted inhibitor of osteoblast Wnt signaling pathway and hypothesized to be a central regulator of prostate cancer osteoblastic bone metastasis. The purpose of this proposal is to examine mechanisms of DKK1 regulation by prostate cancer cells and determine whether DKK1 overexpression in bone blocks the formation of osteoblastic bone lesions in animal models of bone metastasis. We have now shown that human prostate cancer cell lines that produce osteolytic, but not osteoblastic, bone lesions in animal models of bone metastasis express significant amounts of DKK1 and this expression is correlated with the absence of DNA methylation at the DKK1 promoter CpG island. Our preliminary data points to a central role of DKK1 in prostate cancer bone metastasis and expect this work to translate into the development of novel therapeutic targets to treat this malignancy complication.

15. SUBJECT TERMS
Prostate cancer, Bone metastasis, Dickkopf homolog 1, Wnt signaling

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Table of Contents

INTRODUCTION

Osteoblastic bone metastasis is a common complication of advanced prostate cancer, resulting in pain and pathologic fracture (*1*). In mouse models and human clinical studies of prostate cancer, tumor-produced endothelin-1 (ET-1) activates the osteoblast endothelin A receptor and increases new bone formation (*2*). In previously published work from our group, we demonstrated that dickkopf homolog 1 (DKK1), a negative canonical Wnt signaling regulator, is reduced by ET-1 resulting in enhanced canonical Wnt signaling activity and new bone formation (*3*). Others have shown that DKK1 secretion from prostate cancer cells themselves also contribute to bone microenvironment DKK1 (*4*). We hypothesized that DKK1 is a central regulator of prostate cancer bone metastasis. The purpose of this proposal is to examine mechanisms of DKK1 regulation by prostate cancer cells and determine whether DKK1 overexpression in bone blocks the formation of osteoblastic bone lesions in animal models of bone metastasis. Understanding the role of DKK1 in bone metastasis will facilitate the development of modulators of this factor and other Wnt signaling members. The development of such novel and targeted therapies to bone would represent a significant advancement in the treatment of prostate cancer metastasis to bone.

BODY

Task 1: Determine if the osteoblastic response to ET-1 is blocked by *Dkk1* transgenic overexpression targeted to bone in mouse models of prostate cancer bone metastasis

Reliable and reproducible mouse models of bone metastasis utilize the Balb/C nude strain of mice. Immunodeficient nude mice are necessary to avoid rejection of the well-characterized human cancer cell lines. In this task, mouse that overexpress osteoblast DKK1 will be bred to nude mice to test whether increased DKK1 in the bone microenvironment will block the formation of osteoblastic lesions. Since the osteoblast DKK1 overexpressing mice are in the C57Bl/6 strain and will be bred to C57Bl/6 nude animals, we performed a pilot experiment to determine whether LuCaP23.1 and ZR-75-1 cells form osteoblastic lesions similarly in C57Bl/6 vs. Balb/C animals. This pilot experiment is ongoing with results expected in 6 weeks.

Task 2: Determine how DKK1 production from bone cells and tumor is regulated *in vivo* in osteoblastic bone metastasis

The work proposed in this task are dependent on Task 1 and are scheduled to be performed in years two and three of this proposal.

Task 3: Determine if *Dkk1* is inactivated by promoter CpG island hypermethylation in prostate cancer

Significant progress has been made in examining whether DKK1 expression correlates with behavior in bone (osteoblastic vs. osteolytic) and uncovering mechanisms of DKK1 expression by promoter methylation. DKK1 is a secreted inhibitor of the Wnt signaling pathway critical for normal osteoblast differentiation. We

hypothesized that prostate cancer cells with high DKK1 secretion will have suppressed osteoblast new bone formation, tipping the balance towards osteoclastic bone resorption and bone osteolysis. DKK1 expression analysis was performed on prostate cancer cells. The prostate cancer cell line PC3, which produces large osteolytic lesions in an animal model of bone metastasis, exhibited marked DKK1 production. Conversely, C4-2B, C4-2 and LnCaP cell lines, which exhibit osteoblastic/mixed lesions, displayed essentially no DKK1 expression (**Fig. 1**). Methylation-specific PCR was performed on these cell lines to determine the methylation status of DKK1. DNA methylation is an epigenetic mechanism to downregulate gene expression. PC3 cells showed no methylation of the promoter while the other prostate cancer cell lines had some degree of methylation (**Fig. 2**). Promoter methylation is therefore correlated with both DKK1 expression and bone metastasis phenotype.

We next examined DKK1 promoter methylation in human prostate cancer bone metastasis samples. These samples were obtained from our collaborator, Dr. Robert Vessella at the University of Washington. DNA was extracted from fixed paraffin-embedded sections, followed by bisulfite conversion and PCR amplification. However after multiple attempts to sequence the PCR product, quality sequence reads were unsuccessful. These difficulties are the likely consequence of poor bisulfite conversion using small quantities of DNA extracted from the paraffin sections. We will solve this problem by cloning the PCR products into sequencing vectors and DNA sequence directly from these clones. Once we have perfected this technique, additional prostate cancer bone metastasis samples will be obtained from other collaborators as stated in the proposal.

KEY RESEARCH ACCOMPLISHMENTS

- Osteolytic, but not osteoblastic, prostate cancer cells express DKK1
- Osteolytic, but not osteoblastic, prostate cancer cells have unmethylated DKK1 promoter

REPORTABLE OUTCOMES

Oral Presentation, 30th Annual Meeting of the American Society for Bone and Mineral Research, Montreal, 2008.

DKK1 is Regulated by mRNA Stability and Epigenetic Mechanisms in Bone Metastasis
K.L. Clines, J.M. Chirgwin, T.A. Guise, and G.A. Clines
Department of Medicine, University of Virginia

Osteoblastic bone metastasis is a complication of advanced prostate and breast cancer, resulting in pain and pathologic fracture. Tumor-produced endothelin-1 (ET-1) activates the osteoblast endothelin A receptor and increases pathologic new bone formation. Dickkopf homolog 1 (DKK1), a secreted negative regulator of canonical Wnt signaling, is a target of ET-1. ET-1 reduces Dkk1 mRNA and protein secretion in murine primary osteoblasts. Human cancer cells in bone secrete DKK1, whose concentrations are inversely related to osteoblast activity in bone metastasis. We hypothesize that osteoblastic bone metastases depend on decreased DKK1 secretion from two sources in the microenvironment: from

osteoblasts and from tumor cells. ET-1 suppresses osteoblast DKK1 expression, while tumor expression of DKK1 is not well understood.

DKK1 down-regulation by ET-1 in the osteoblast was explored using *Dkk1*/luciferase reporter constructs. ET-1 did not change *Dkk1* promoter (1.7 kb) activity in murine primary osteoblasts. We then examined whether ET-1 decreases *Dkk1* mRNA stability via the 3'UTR, a 1.3 kb segment containing consensus binding elements for AUF1 and miRNAs. ET-1 significantly reduced (0.52 X, p=0.0005) luciferase activity in a construct containing the *Dkk1* 3'UTR indicating that ET-1 regulates osteoblast *Dkk1* via message stability.

We next examined relative expression of *DKK1* and its CpG island methylation in prostate and breast cancer cell lines. Using real-time RT PCR, we found C4-2B, C4-2, LnCaP prostate and T47D breast lines had low DKK1 expression while ZR-75.1, MCF-7, MDA-MB-231 breast and PC3 prostate lines had significant *DKK1* expression. *DKK1* contains a 5' 233 bp CpG island encompassing the transcriptional start site with 18 potential cytosine methylation sites. Changes in CpG methylation patterns are one mechanism responsible for gene misexpression during tumorigenesis. Methylation-specific sequencing of the *DKK1* CpG island was performed on the human cancer cells. Methylation was detected in the cancer cell lines with the lowest *DKK1* expression while the sequence was unmethylated in cells with the highest *DKK1* expression.

These observations are consistent with a model for osteoblastic bone metastases in which DKK1 production is decreased in both cancer cells and osteoblasts. The mechanisms involve both static epigenetic promoter silencing and dynamic control of *DKK1* mRNA stability via its 3'UTR. *DKK1* mRNA stability can be regulated by tumor-produced ET-1, while the factors that control its basal promoter activity via CpG island methylation are presently unknown. Understanding the regulation of *DKK1* gene expression in tumor and bone may lead to novel therapies for osteoblastic metastases.

CONCLUSION

Bone metastasis is a significant complication of advanced prostate cancer that causes pain and pathologic fracture. This work is aimed at uncovering the role of DKK1 in prostate cancer bone metastasis. In this initial year of proposal funding, we have discovered a correlation between behavior of prostate cancer in bone, DKK1 expression and DNA methylation of the DKK1 promoter. We will extend this work and examine DKK1 promoter methylation patterns in human prostate cancer bone metastasis and whether this pattern correlates with risk for and progression of bone metastasis. In the second year of this proposal, we will examine whether overexpression of DKK1 in the bone microenvironment blocks bone metastasis in an animal model. Medical and social costs of bone metastasis are high. This work is expected to translate into improved treatments for prostate cancer bone metastasis and facilitate the development of therapeutic targets to DKK1.

REFERENCES

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2. J. J. Yin *et al.*, *Proceedings of the National Academy of Sciences of the United States of America* **100**, 10954 (2003).
3. G. A. Clines *et al.*, *Molecular Endocrinology* **22**, 486 (2007).
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APPENDICES

None

SUPPORTING DATA

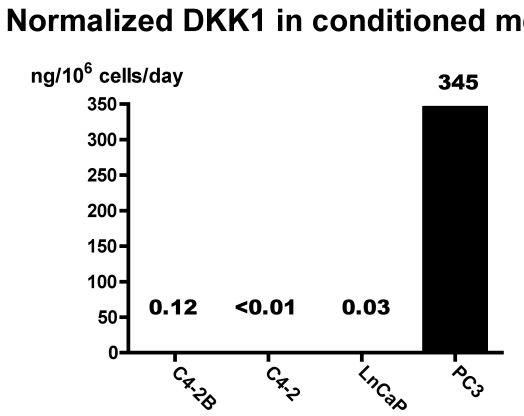

Fig. 1. DKK1 secretion correlates with prostate cancer bone metastasis phenotype. C4-2B, C4-2, LnCaP and PC3 human prostate cancer cell lines were grown in culture and conditioned media was collected for DKK1 ELISA analysis. DKK1 concentration was normalized to cell number. The osteolytic cancer cell line PC3 exhibited marked DKK1 secretion.

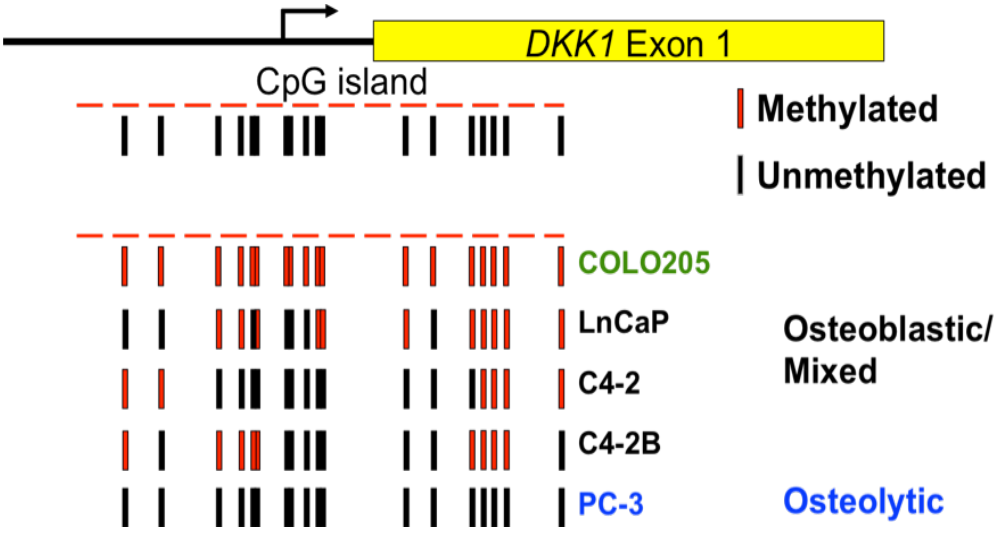

Fig. 2. DKK1 promoter methylation correlates with prostate cancer bone metastasis phenotype. The DKK1 promoter contains a 233 bp CpG island with 18 potential methylation sites (bars). Methylation-specific sequencing of this CpG island was performed in human cell lines. The COLO205 colon cancer cell line served as a control for methylation. The prostate cancer cell lines that produce osteoblastic or mixed lesions (LnCaP, C4-2, C4-2B) displayed different degrees and patterns of methylation. The osteolytic cell line PC3 displayed no methylation.